PLATYPUS TECHNOLOGIES

Bringing Science to the Surface™

Oris™ Collagen I Cell Invasion Assay

Product No.: CIA101CC & CIA200CC

96-well, 3-D Assay for Investigating Cell Invasion
of Adherent Cell Lines on Collagen I

PROTOCOL & INSTRUCTIONS

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ORIS™ COLLAGEN I CELL INVASION ASSAY

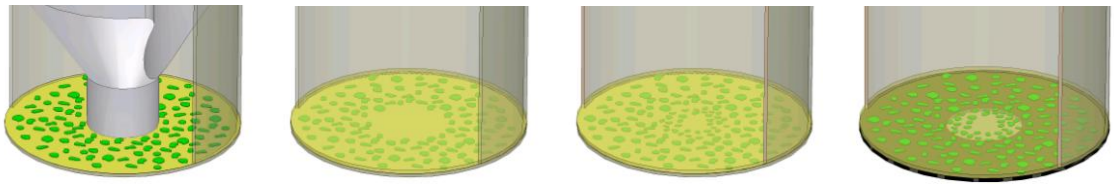
I. INTRODUCTION

The Oris™ Collagen I Cell Invasion Assay is a reproducible, sensitive, and flexible assay that can be used to monitor cell invasion. Formatted for a 96-well plate, the assay utilizes Oris™ Cell Seeding Stoppers made from a medical-grade silicone to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2 mm diameter unseeded region in the center of each well, i.e., the detection zone, into which the seeded cells may then invade once the Collagen I Overlay has been applied. The Oris™ Detection Mask is applied to the plate bottom and restricts visualization to the detection zone, thus allowing only motile cells to be detected (see Figure 1). The Oris™ Collagen I Cell Invasion Assay is designed to be used with any commercially available stain or labeling technique. The readout can be performed by using a microscope, a microplate reader, or a High Content Screening or High Content Imaging Analysis platform.

The Oris™ Collagen I Cell Invasion Assay kit has been uniquely designed to detect cellular migration and invasion *in vitro* within a 3-dimensional extracellular matrix comprised of Collagen I (rat-tail). The Oris™ Collagen I Cell Invasion Assay system has been designed for use with adherent cell cultures. Performance of the assay was optimized using HT-1080 fibrosarcoma and MDA-MB-231 breast epithelial cell lines.

Using the Oris™ Collagen I Cell Invasion Assay offers the following features & benefits:

- **Membrane-free Invasion** - perform studies without manipulating transmembrane inserts; no membrane to restrict the ability to image cells.
- **Reproducible Results** – obtain low well-to-well CV's due to the unique assay design.
- **Preserve Cell Morphology** – realize a more native 3-D environment.
- **Versatile** - analyze data using multiple probes in a single well by using a microscope, digital imager, or fluorescence microplate reader.
- **Flexible** - perform real-time or endpoint cell invasion assays without the use of special instrumentation.



Seed & Adhere Cells onto Oris™ Collagen I Coated Plate

Remove Stoppers to Create Detection Zone & Apply Collagen I Overlay

Incubate and Allow Cells to Invade into Detection Zone

Analyze Cells in Detection Zone: Microplate Reader Analysis - Detection Mask Attached; Image Analysis - No Mask Required

Figure 1. Schematic of Oris™ Collagen I Cell Invasion Assay

II. ORIS™ PLATE DIMENSIONS

Diameter of Well	6.5 mm
Diameter of Stopper Space (Detection Zone)	2 mm
Suggested Media Volume per Well (populated with Stoppers)	100 µL
Effective Area of Outer Annular Region (seeding region) per Well	30.03 mm^2
Effective Area of Central Detection Zone per Well	3.14 mm^2
Plate Height	14.9 mm
Plate Height with Lid (with Oris™ Cell Seeding Stoppers)	23.9 mm
Offset of Wells (A-1 location, X)	14.4 mm
Offset of Wells (A-1 location, Y)	11.2 mm
Distance between Wells	9 mm (on center)
Well Depth	12.2 mm
Thickness of Well Bottom	0.25 mm
Well Coating Material	Collagen I, rat-tail
Storage Conditions	Refrigerate (4°C)

Important: Read Instructions Before Performing any Oris™ Assay.

III. MATERIALS PROVIDED

Product No.: CIA101CC

Component	Quantity	Storage
Oris™ Collagen I Coated, 96-well Plate with Oris™ Cell Seeding Stoppers	1	Refrigerate (4°C)
Oris™ Detection Mask	1	Room Temperature
Oris™ Stopper Tool	1	Room Temperature
Oris™ Collagen I Stock Reagent *	4 mL	Refrigerate (4°C)
Oris™ 10X PBS Buffer	1 mL	Refrigerate (4°C) / Room Temperature

Product No.: CIA200CC

Component	Quantity	Storage
Oris™ Collagen I Coated, 96-well Plates with Oris™ Cell Seeding Stoppers	2	Refrigerate (4°C)
Oris™ Detection Mask	1	Room Temperature
Oris™ Stopper Tool	1	Room Temperature
Oris™ Collagen I Stock Reagent *	2 x 4 mL	Refrigerate (4°C)
Oris™ 10X PBS Buffer	2 x 1 mL	Refrigerate (4°C) / Room Temperature

* Oris™ Collagen I Stock Reagent must be stored at 4°C for use within 6 months of receipt. ***Do not freeze.***

Oris™ is a trademark of Platypus Technologies, LLC.

IV. MATERIALS REQUIRED

- Biological Cells
- 7.5% Sodium Bicarbonate
- Complete Cell Culture Growth Medium (containing serum)
- Sterile PBS (containing both Ca^{++} and Mg^{++})
- Hanks Balanced Salt Solution (HBSS)
- Serum-Free Cell Culture Medium
- Sterile Pipette Tips/Pipette or Multi-Channel Pipette
- Trypsin or Cell Scraper
- Inverted Microscope (optional)
- Fluorescence Microplate Reader (optional)
- Cell Culture Labeling Medium (phenol red-free/serum-free media)
- Cell Labeling Fluorescent Agent (e.g., Calcein AM) - *required if performing staining.*

V. PRECAUTIONS AND RECOMMENDATIONS

For Research Use Only. Not for use in diagnostic procedures.

Handling and Use of the *Oris™ Collagen I Stock Reagent*:

- Please note that it is crucial that the Collagen I Overlay concentration be optimized for cell line and experimental conditions, since different cell lines and different experimental conditions can result in varied amounts of cell invasion.
- A suggested starting concentration for the ***Oris™ Collagen I Overlay*** is 3 mg/mL.

Recommendations for Preparation of Reference Wells:

- To establish t=0 pre-invasion reference wells by seeding both test and reference wells at the ***same time***, it is necessary to seed cells at ***different concentrations***. Seed test wells at a density determined optimal in Appendix I, but seed reference wells at sub-optimal density (50-75% confluency). Allow cells to adhere in all wells and remove stoppers from test wells (treat test wells with the Oris™ Collagen I Overlay, incubate to allow for gel formation, and proceed with invasion experiment). Reference wells will remain populated with Oris™ Cell Seeding Stoppers until the end of the assay. At that point, remove the Oris™ Cell Seeding Stoppers from the reference wells, treat the wells with the Oris™ Collagen I Overlay, incubate to allow for gel formation and proceed with staining/analysis of the entire plate.

- To establish t=0 pre-invasion reference wells by seeding test and reference wells at the ***same concentration***, it is necessary to seed cells at ***different times*** during the assay. Seed test wells at density determined optimal in Appendix I, allow cells to adhere for 1 – 18 hours, remove stoppers, treat with the Oris™ Collagen I Overlay, and incubate to allow for gel formation. Allow cells to invade for a set amount of time. At 1 - 18 hours prior to analyzing test wells, seed reference wells at a density determined optimal in Appendix I, and allow cells to adhere. At the end of the assay, remove stoppers from the reference wells, treat with the Oris™ Collagen I Overlay, incubate to allow for gel formation, and proceed with staining/analysis of the entire plate.

Experimental Conditions

- Please note that cell movement along the X-Y axes will likely occur in addition to invasion in the Z-axis. The degree of X-Y movement will vary for different cell lines.

Recommendations for 10X PBS Buffer:

- When 10X PBS is refrigerated, sedimentation may occur due to the high salt concentration. If sediment forms, warm the PBS in a water bath (37 °C) to completely dissolve any sediment prior to use.

VI. COLLAGEN I CELL INVASION ASSAY PROTOCOL

The following steps should be performed in a biological hood using aseptic technique to prevent contamination.

1. If desired, cells can be starved by incubating for 18 - 24 hours in serum-free medium prior to assay (0.5% fetal bovine serum may be used if needed).

2. Remove the Oris™ Collagen I Coated Plate from refrigeration and place on lab bench for ~1 hour to allow it to equilibrate to room temperature.

3. Visually inspect the underside of the populated 96-well plate to ensure that the Oris™ Cell Seeding Stoppers are firmly sealed against the bottom of the plate. To inspect the stoppers, turn the plate over and examine the stoppers for sealing (see Figure 2). If incomplete sealing is observed, return the plate to the upright position and use a sterile instrument to gently push the stopper back into the well until sealing is observed.

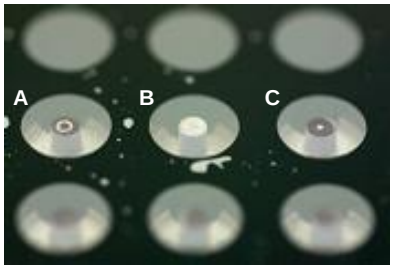


Figure 2. Stoppers that are:
A) Partially Sealed
B) Unsealed
C) Completely Sealed

NOTE: The sealing of the stoppers can be most easily observed if the plate is tipped at an angle and viewed under indirect light to reveal the "bullseye" pattern at the bottom of each well.

4. Apply the Oris™ Detection Mask to the bottom of the 96-well plate if microplate reader data is being collected. The Detection Mask is not necessary if collecting imaging data.

   First Time Users: In order to prevent splashing of well contents, familiarize yourself with the attachment and removal of the Detection Mask before any liquids are placed into the wells:

   - Orient the chamfered corners of the mask with those of the 96-well plate, ensuring that the A1 corner of the mask is aligned with the A1 well of the plate (see Figure 3).
   - Align the holes in the attachment lugs with the bosses on the bottom of the plate.
   - Gently press the mask until it is flush with the bottom of the 96-well plate.

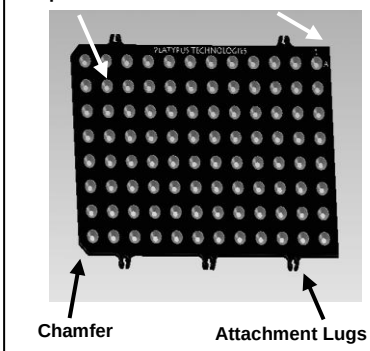


Figure 3. Features of Detection Mask

NOTE: It may be necessary to wash the mask with ethanol to remove dust and debris since the mask is **not** sterile. The mask may be applied at any point during the assay. For kinetic assays, it is often most convenient to apply the mask at the beginning of the assay before any liquids are placed in the well. For endpoint assays, using fixed and stained cells, it is often most convenient to apply the mask just before reading assay results.

5. If performing kinetic analysis of cell invasion, pre-label cells with a fluorescent stain at this time. Refer to Section VII and Appendix II for further information on data acquisition and fluorescent labeling of live cells.

6. Collect cells and prepare a suspension that is 10-fold greater in density than the optimal seeding concentration using complete cell culture growth medium containing serum.

   First Time Users: The optimum seeding density of cells must be determined as an integral part of the design of the cell invasion assay. Please refer to Appendix I for a discussion of this process.

   IMPORTANT: For recommendations on designating 'reference' wells, please refer to Section V: Precautions and Recommendations.

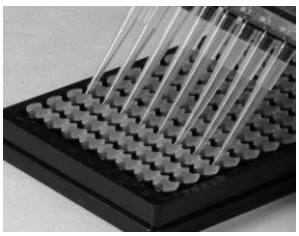


Figure 4. Media is Added with Single or Multi-Channel Pipette

7. Pipette 100 µL of suspended cells into each test well through one of the side ports of the Oris™Cell Seeding Stopper.

NOTE: For best results, add or extract media by placing the pipette tip along the wall of the well (see Figure 4). Care should be taken not to disturb the Oris™ Cell Seeding Stopper or the Collagen I coating when introducing the pipette tip into the well. A slender/elongated tip or a gel loading tip may be useful.

IMPORTANT: Lightly tap the plate on your work surface to evenly distribute well contents (extreme tapping may result in splashing of well contents and lead to contamination).

8. Incubate the seeded plate containing the Oris™ Cell Seeding Stoppers in a humidified chamber (37°C, 5% CO_2) for 1 to 18 hours (cell line dependent) to permit cell attachment.

9. Remove plate from incubator.

10. Using the Oris™ Stopper Tool, remove stoppers (see Figure 5).

NOTE: It may be necessary to wash the Oris™ Stopper Tool with 70% ethanol as the Stopper Tool is not sterile.

- Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Oris™ Stopper Tool under the backbone of the stopper strip, keeping the underside of the Oris™ Stopper Tool flush with the top surface of the plate.
- Lift the Oris™ Stopper Tool ***vertically*** to gently remove stoppers.

Do not use the Oris™ Stopper Tool as a lever to pry the stoppers from the well (see Figure 5E), as doing so may cause displacement of seeded cells and may distort the detection zone area.

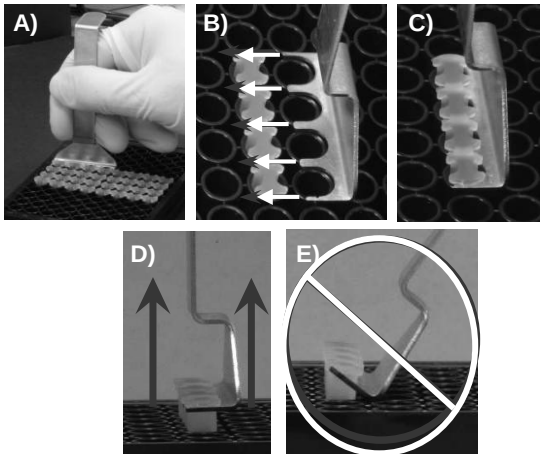


Figure 5. Removal of Stoppers. Panels A, B, and C) Position the Tines of the Stopper Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers

11. Remove media with a pipette and ***gently*** wash wells with 100 µL of serum-free media (or sterile PBS) to remove any unattached cells. Do not aspirate using an in-house vacuum.

IMPORTANT: Prior to/during use, keep the ***Oris™ Collagen I Stock Reagent*** and the ***Oris™ Collagen I Overlay Solution*** on ice. In addition, the use of chilled pipette tips/reservoirs might be beneficial.

12. Prepare 5.0 mL of an appropriate concentration of the ***Oris™ Collagen I Overlay*** solution, using the following components:
    - Oris™10X PBS (sterile)
    - 7.5% sodium bicarbonate (sterile)
    - Deionized water (sterile)
    - ***Oris™ Collagen I Stock Reagent*** (5 mg/mL)

Calculate the volume of ***Oris™ Collagen I Stock Reagent*** needed to make the desired concentration of the ***Oris™ Collagen I Overlay*** solution. Calculate the volume of sodium bicarbonate needed to neutralize the collagen where 0.0125ml of 7.5% sodium bicarbonate is required for every 1 mL of 5 mg/mL ***Oris™ Collagen I Stock Reagent*** used. Appropriate volumes of 10X PBS and deionized water are used to prepare the Collagen I overlay in a final 1X PBS solution.

On ice, combine the water, Oris™ 10X PBS, and sodium bicarbonate. Next, add the ***Oris™ Collagen I Stock Reagent*** to achieve the desired concentration of the Collagen I Overlay solution.

The following example protocol provides volumes for a 3.0 mg/mL Collagen I Overlay solution:

1.4625 mL deionized water
0.5 mL Oris™ 10X PBS buffer
0.0375 mL 7.5% sodium bicarbonate
3 mL ***Oris™ Collagen I Stock Reagent*** (5mg/mL)
5.0 mL total volume

NOTE: Supplements, such as growth factors, may be mixed with the 3-D Collagen I Overlay.

13. Remove media from wells and add 40 µL of the ***Oris™ Collagen I Overlay*** to each well.

IMPORTANT: Place plate on ice during addition of the ***Oris™ Collagen I Overlay*** to reduce premature polymerization of the Collagen I.

14. Incubate plate in a humidified chamber (37°C, 5% CO_2) for 1 hour to permit polymerization of the 3-D Collagen I overlay.

15. Add 100 µL of complete media (containing serum) on top of the 3-D Collagen I Overlay. **Optional**: Invasion inhibitors or stimulants may be added to the media.

IMPORTANT: Use caution when adding media so as not to dislodge the Collagen I Overlay from bottom/sides of the well.

16. Incubate plate in a humidified chamber (37°C, 5% CO_2) to permit cell invasion (length of incubation is cell line dependent). Refresh media or supplements, every 48 - 72 hours, as needed, for the duration of the invasion experiment.

17. If performing an endpoint analysis of cell invasion, stain cells with a fluorescent stain after sufficient invasion has occurred. Refer to Section VII and Appendices II & III for further information on data acquisition and fluorescence staining technique.

NOTE: Oris™ Cell Seeding Stoppers are for single use only; Platypus cannot guarantee the integrity of the stopper material after a second sterilization procedure.

VII. DATA ACQUISITION

The readout of the Oris™ Collagen I Cell Invasion Assay can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Collagen I Cell Invasion Assay is designed to be used with any commercially available stain or labeling technique. The readout can be performed by using a microscope, a microplate reader, or a High Content Screening or High Content Imaging instrument.

Microscope Analysis

- Cell counting or image analysis software, such as NIH ImageJ freeware, can be used.
- Note: Microscopic observations are possible using phase contrast or fluorescence microscopy.
- No need to attach the Oris™ Detection Mask to the Oris™ microplate.
- To set up reference controls, refer to Section V: Precautions and Recommendations.

Microplate Reader Analysis

- Attach the Oris™ Detection Mask to the bottom of the Oris™ microplate (see Step 4 of Protocol).
- Optimal settings will vary according to the microplate reader make and model. Consult Appendix II and the equipment user manual for your particular instrument.
- The microplate reader MUST be set to read from the bottom of the plate.
- To set up reference controls, refer to Section V: Precautions and Recommendations.

Sample Data Obtained via Microscopy and Microplate Reader are shown in Figure 5.

- Wells were seeded with 30,000 HT-1080 cells (i.e., 100 µL of $3.0x10^5$ cells/mL) and the plate was incubated for 1 hour. The stoppers were removed from the wells, the wells were rinsed with serum-free media, and the Oris™ Collagen I Overlay without serum (final concentration of 3 mg/mL) was overlayed on the cell monolayer (sixteen (16) wells were left stoppered to represent t=0 reference). After polymerization was permitted for 1 hour, complete media (containing 10% FBS) was added on top of the Collagen I Overlay. The plate was then incubated in a humidified chamber for 48 hours to permit cell invasion. Cells were labeled with Calcein AM and images were captured using a Zeiss Axiovert microscope (5X magnification). Fluorescence in the detection zone was quantified by using a microplate reader. The images below, captured without a detection mask in place, illustrate representative data from pre-invasion, t=0 hrs (image 1a) and post-invasion, t=48 hrs (image 1b) wells. The graph depicts the average Relative Fluorescent Units (RFU's) in the detection zones for each condition (each column represents the mean +/- SD of at least 16 wells).

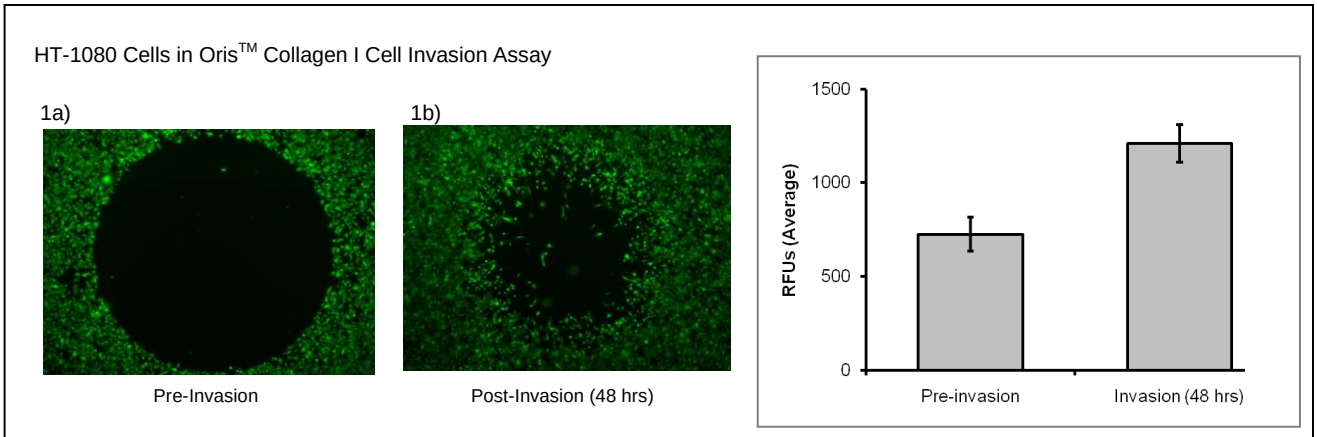


Figure 5. Cell invasion data obtained via microscopy and microplate reader analysis

VIII. ORDERING INFORMATION

Product Name	Coating	Size	Detection Zone Format
Oris™ Collagen I Cell Invasion Assay	Collagen I	1-pack (CIA101CC) 2-pack (CIA200CC)	Oris™ Cell Seeding Stoppers (pre-populated)
Oris™ Cell Invasion & Detection Assay	BME	1-pack (CIA101DE) 2-pack (CIA200DE)	Oris™ Cell Seeding Stoppers (not pre-populated)
Oris™ Pro Cell Migration Assays	Tissue Culture Treated	1-pack (PROCMA1) 5-pack (PROCMA5)	Biocompatible Gel
	Collagen I Coated	1-pack (PROCMACC1) 5-pack (PROCMACC5)	
Oris™ Cell Migration Assays	Tissue Culture Treated	1-pack (CMA1.101) 5-pack (CMA5.101)	Oris™ Cell Seeding Stoppers (pre-populated)
	Collagen I Coated	1-pack (CMACC1.101) 5-pack (CMACC5.101)	
	Fibronectin Coated	1-pack (CMAFN1.101) 5-pack (CMAFN5.101)	
	TriCoated	1-pack (CMATR1.101) 5-pack (CMATR5.101)	
Oris™ Cell Migration Assembly Kits	Universal (Tissue Culture Treated)	1-pack (CMAU101) 5-pack (CMAU505)	Oris™ Cell Seeding Stoppers (not pre-populated)
	Collagen I Coated	1-pack (CMAUCC1) 5-pack (CMAUCC5)	
	FLEX (Tissue Culture Treated)	4-pack (CMAUFL4)	

For a complete list of assays, visit Platypus Technologies at www.platypustech.com/order_main.html.

For technical assistance, contact Technical Support at (866) 296-4455 or techsupport@platypustech.com.

IX. TERMS & CONDITIONS

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This warranty shall not be effective if PLATYPUS determines, in its sole discretion that PURCHASER has altered or misused the goods or has failed to use or store them in accordance with instructions furnished by PLATYPUS. PLATYPUS's sole and exclusive liability and PURCHASER's exclusive remedy with respect to goods proved to PLATYPUS's satisfaction (applying analytical methods reasonably selected by PLATYPUS) to be defective or nonconforming shall be the replacement of such goods free of charge, upon the return of such goods in accordance with our instructions, although at its discretion, PLATYPUS may provide a credit or refund. If PLATYPUS manufactures custom goods for PURCHASER based on instructions, specifications, or other directions provided by PURCHASER, PLATYPUS shall not be liable for the lack of sufficiency, fitness for purpose or quality of the goods to the extent attributable to such instructions, specifications, or other directions. PLATYPUS shall not be liable for any loss, damage or penalty as a result of any delay in or failure to manufacture, deliver or otherwise perform hereunder due to any cause beyond PLATYPUS's reasonable control.

PLATYPUS shall not be liable for injury or damages resulting from the use or misuse of any of its products.

APPENDIX I: Determining Optimal Cell Seeding Concentration

This procedure is intended to assist in determining the cell seeding density needed to achieve confluency of your cell line when using the Oris™ Collagen I Cell Invasion Assay. The intended goal is to achieve 90-95% confluency of the monolayer surrounding the Oris™ Cell Seeding Stoppers without overgrowth.

1. A suggested starting point is to evaluate three serial dilutions at the cell densities shown below. The cell seeding area of the well with the stopper in place is ~ 0.3 cm^2. Based on the typical seeding density of your particular cell line, you can infer a different cell number for your first serial dilution and adjust the numbers below accordingly.
2. Prepare a log-phase culture of the cell line to be tested. Collect cells and determine the total number of cells present.
3. Pellet cells by centrifugation. Prepare three serial dilutions at final concentrations of 1.0 x 10^6, 0.5 x 10^6 and 0.25 x 10^6 cells/mL.
4. Dispense 100 µL of cell suspension per well into the 96-well plate to result in the following plate layout:

Column	1	2	3
Cells / well	100,000	50,000	25,000
Number of wells	8	8	8

5. Incubate the plate in a humidified chamber (37°C, 5% CO_2) for 1 - 18 hours (cell line dependent) with cell seeding stoppers in place to allow the cells to firmly attach to the well surface.
6. Following cell attachment, remove the Oris™ Cell Seeding Stoppers from each well (see Figure 6) and ***gently*** wash the wells with PBS to remove non-attached cells.
   - Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Oris™ Stopper Tool under the backbone of the stopper strip, keeping the underside of the tool flush with the top surface of the plate.
   - Lift the Oris™ Stopper Tool ***vertically*** to gently remove the stopper. Do not use the Oris™ Stopper Tool as a lever to pry the stoppers from the well as doing so may cause displacement of the seeded cells.
7. Without a Detection Mask in place, use a microscope to visually inspect each well to determine the minimum cell seeding concentration that yielded a confluent monolayer at the perimeter of the detection zone.

At this point, if you plan to obtain the results of the Oris™ Collagen I Cell Invasion Assay via colorimetric analysis or microscopy, you have successfully determined the optimal cell seeding concentration to be used in Step 6 of the Oris™ Collagen I Cell Invasion Assay Protocol.

APPENDIX II: Fluorescent Labeling Live Cell Options

This procedure is intended to assist in obtaining data from the Oris™ Collagen I Cell Invasion Assay using various fluorescent labels.

The Oris™ Collagen I Cell Invasion Assay has been designed to work with all types of fluorescent stains and staining techniques. The precise method for staining cells with fluorescent stains varies according to the nature of the individual stain. It is important to stain cells using a fluorescent reagent that uniformly stains cells. Probes affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Please consult the manufacturer of your fluorescent stain for specific considerations.

NOTE: Use caution when adding/removing solutions so that the Collagen I Overlay is not dislodged from the bottom/sides of the well.

The following is an example Fluorescent Staining Protocol to label live cells with Calcein AM:

a) To stain one fully-seeded 96-well plate, combine 5 µL of Calcein AM (1 mg/mL in dry DMSO) with 10 mL of phenol red-free and serum-free media or 1x PBS (containing both Ca^{++} and Mg^{++}). Protect diluted Calcein AM solution from light until ready to use in step d.
b) Carefully remove culture medium from wells.
c) Wash wells with 100 µL of PBS (containing both Ca^{++} and Mg^{++}).
d) Add 100 µL of diluted Calcein AM solution to each well.
e) Incubate plate at 37°C for 30 - 60 minutes.
f) Attach mask and read promptly with microplate reader using appropriate filter set and sensitivity/gain settings (for a BioTek Synergy™ HT microplate reader, use 485/528 nm excitation/emission filters, sensitivity 55 nm).

If not already in place, apply the Oris™ Detection Mask to the plate. Using the bottom probe of a fluorescence microplate reader, obtain the fluorescence reading from each well. To achieve the optimal dynamic range, adjust the instrument settings (e.g., gain) to result in the greatest difference in fluorescence signal between pre-invasion and post-invasion wells. Refer to the instrument manual for your microplate reader for further guidance on instrument settings.

At this point, you have successfully labeled your live cells.

APPENDIX III: Fluorescent Labeling Fixed Cell Options

This procedure is intended to assist in obtaining data from the Oris™ Collagen I Cell Invasion Assay using various fluorescent labels.

The Oris™ Collagen I Cell Invasion Assay has been designed to work with all types of fluorescent stains and staining techniques. The precise method for treating cells with fluorescent stains varies according to the nature of the individual reagent. It is important to use a fluorescent reagent that uniformly stains cells. Probes affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Please consult the manufacturer of your fluorescent stain for specific considerations.

NOTE: Use caution when adding/removing solutions so that the Collagen I Overlay is not dislodged from the bottom/sides of the well.

The following is an example Fluorescent Staining Protocol to label fixed cells with TRITC-phalloidin (F-actin) and DAPI (nuclei):

a) To fix one fully-seeded 96-well plate, prepare 10 mL of fixative solution (e.g., 0.25% glutaraldehyde solution in PBS prepared from 8% glutaraldehyde solution (Electron Microscopy Sciences)).
b) Remove media and rinse wells with 100 µL of PBS.
c) Remove PBS and add 100 µL of a fixative solution (0.25% glutaraldehyde solution in PBS) to each well and incubate at room temperature for 15 minutes.
d) Remove fixative solution and rinse wells with 100 µL of PBS.
e) Remove PBS and replace with 100 µL of a 1:50-1:100 dilution of TRITC-phalloidin (Sigma; prepared as 10 µM stock in methanol) in PBS containing 0.1% Triton X-100.
f) Incubate plate at room temperature for 45 minutes (protect from light).
g) Remove the TRITC-phalloidin and add 100 µL of a 1:4000 dilution of DAPI (ThermoScientific) in PBS.
h) Incubate plate at room temperature for 2-10 minutes (protect from light).
i) Remove DAPI stain and wash wells 2x for 5 minutes each with 200 µL of PBS.
j) Replace final wash with 200 µL of fresh PBS.

NOTE: This protocol outlines double-labeling of cells with a cytoskeletal and a nuclear stain. The protocol can be simplified if only one stain is used. Substitutions or additional cytostaining or immunostaining may be performed using non-overlapping fluorophores and by utilizing the appropriate filters with your imaging equipment.

At this point, you have successfully fixed and labeled your cells.